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Comparison of Killer Ig-Like Receptor Genotyping and Phenotyping for Selection of Allogeneic Blood Stem Cell Donors

Wing Leung,†‡ Rekha Iyengar,† Brandon Triplett,† Victoria Turner,‡ Frederick G. Behm,‡ Marti S. Holladay,† James Houston,† and Rupert Handgretinger†‡

The repertoire of killer Ig-like receptors (KIRs) can be determined at the level of DNA, RNA, or surface protein expression for selection of blood stem cell donors. We compared genotyping and phenotyping of the four inhibitory KIRs that are important in transplantation for leukemia in 73 unrelated persons. In 5 (7%) of the 68 individuals in whom the KIR2DL1 gene was present and in 10 (15%) of the 67 in whom KIR3DL1 was present, the corresponding receptor was not expressed by NK cells, as determined by flow cytometry analysis. In contrast, one or both allelic forms of KIR2DL2/KIR2DL3 were expressed by a high proportion of NK cells in all 73 individuals. However if both KIR2DL2 and KIR2DL3 genes were present, KIR2DL3 was preferentially expressed, as transcripts of KIR2DL2 was not detectable by RT-PCR in 42% of these individuals. In total, repertoire assessment for the four KIRs by genotyping vs phenotyping was not in complete agreement in 18 (25%) of the 73 individuals. Furthermore, among the samples that tested positive for the expression of a certain KIR gene, the levels of transcripts and surface expression varied considerably as measured by both real-time quantitative PCR and flow cytometry analysis. Extension of this comparative analysis to include all 12 KIR family members showed that KIR2DL3 and KIR3DL2 were the only genes whose transcripts were consistently detectable. These results caution the use of genotyping alone for donor selection or leukemia-relapse prognostication because some KIRs may be expressed at a very low level. The Journal of Immunology, 2005, 174: 6540–6545.

Human NK cells are regulated by killer Ig-like receptors (KIRs) that recognize specific groups of HLA class I alleles. The genomic region that encodes KIRs exhibits extensive variability among individuals due to differences in gene content, gene copy number, and allelic polymorphism (1, 2). Thus, two unrelated persons almost always have different KIR genotypes. Because the genes that encode HLA and KIR segregate independently, the likelihood of KIR disparity approaches 100% in allogeneic hematopoietic stem cell transplantation (HCT) between unrelated individuals and exceeds 75% between family members regardless of HLA identity. The clonal repertoire of NK cells is diversified further by the apparently stochastic combinatorial nature of KIR expression (3).

Many clinical studies (4–6), but not all (7–9), have shown the importance of KIR mismatch between donor and recipient in improving the outcomes of HCT for leukemia. Besides the confounding effects of T cell alloreactivity (10) and antithymocyte globulin (11), another reason for the conflicting results is the difference in definition of “KIR mismatch.” For instance, KIR mismatch was defined by the Perugia group as mismatch between the donor KIR ligand and recipient KIR ligand (4) and by the Nantes group as mismatch between the donor KIR and recipient KIR (7). Recently, we showed that the risk of leukemia relapse was best prognosticated by a third definition, a mismatch between the donor KIR and recipient KIR ligand (12), i.e., missing ligand in the recipient for the donor inhibitory KIRs. Only KIR-KIR ligand interactions have been demonstrated to be biologically relevant thus far; there are no known interactions between KIR ligands on NK cells and KIR ligands on target cells, or between KIR on NK cells and KIR on target cells. In addition, the ligands on target cells and those on NK cells have minimal influence on the KIR repertoire of NK cells before and after HCT (12–14). It has been shown that within 3 mo after HCT without graft-vs-host disease (GVHD) prophylaxis, NK cells derived from a donor’s CD34+ cells normally acquired a donor-specific pattern of KIR expression that was independent of the donor’s or recipient’s HLA repertoire (12). Taken together, these results suggest that the “best KIR mismatch” donor could be selected on the basis of a single, direct measurement of the donor’s KIR repertoire and recipient’s ligand repertoire before HCT.

In addition to the difference in the definition of KIR mismatch, another level of complexity involves the method of KIR typing. At present, donor KIR repertoire can be determined by flow cytometry for surface expression, by RT-PCR using sequence-specific primers (SSP) for mRNA expression, or by PCR-SSP for genotyping. The optimal method of KIR analysis for donor selection has not been established. Herein, we report the results from a comparative genotyping and phenotyping study that caution the use of genotyping alone in the assessment of donor KIR repertoire.

Materials and Methods

KIR nomenclature and typing

The nomenclature for KIR genes and proteins followed those of Marsh et al. (15). KIR3DL1 is specific for HLA-B allotypes expressing the serologic

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† Abbreviations used in this paper: KIR, killer cell Ig-like receptor; HCT, hematopoietic stem cell transplantation; GVHD, graft-versus-host disease; RQ-PCR, real-time quantitative PCR; TMA, trichostatin A; 5AzadC; 5-aza-2’-deoxycytidine.
Bw4 epitope; KIR2DL1 is specific for HLA-C allotypes with a lysine residue at position 80 (HLA-CLys80) of the α1 helix, and KIR2DL2 and KIR2DL3 are both specific for HLA-B*4601 and HLA-C allotypes with asparagine at position 80 (HLA-CAsn80). Surface expression of these four KIRs by NK cells was determined by flow cytometry analysis using mAbs as described (12): KIR3DL1 was detected by DX9 (BD Immunocytometry Systems) (16–18), KIR2DL1 by EB6B (Immunotech) (19, 20) and HP-3E4 (BD Immunocytometry Systems) (21), and KIR2DL2/KIR2DL3 by CH-L (BD Pharmingen) (22), GL183 (Immunotech) (19, 22), and DX27 (BD Pharmingen) (17).

Transcripts of KIR genes were detected by using RT-PCR analysis as described by Uhrberg et al. (23). KIR2DL5 and 3DL3 were not included in this report because their transcripts may not be reliably detected by standard RT-PCR (3, 24). KIR genotyping was done by using PCR analysis as described previously (12) and with the KIR Genotyping kit from Pel-Freez per the manufacturer’s instructions.

Quantification of KIR transcripts

Real-time quantitative PCR (RQ-PCR) was performed by using the ABI Prism 7700 Sequence Detector Systems (Applied Biosystems) and the SYBR Green I Dye assay chemistry, as suggested by the manufacturer. Briefly, all reactions were performed with 2 μl (80 ng) of cDNA, 12.5 μl of SYBR GREEN PCR master mix (Applied Biosystems), and 0.5 μM forward and reverse primers in a final reaction volume of 25 μl. Primer sets used were the same as those of standard PCR. Cycling parameters were 95°C for 10 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s, followed by extension at 72°C for 3 min. SYBR Green I binds to the minor groove of dsDNA. During consecutive PCR cycles, the amount of double-stranded PCR products increased exponentially; therefore, more SYBR Green I dye bound and emitted fluorescence (at 520 nm), which was detectable by the charge-coupled device camera. A water control and melting curve analysis were always performed to confirm the specificity of the PCR. GAPDH was used as an internal control to normalize the difference in the amount of cDNA contained in each initial reaction. For quality control purposes, RQ-PCR analysis was performed on 27 samples that were negative for a particular KIR by genotyping and standard RT-PCR methods; results were also negative in all cases (data not shown).

Induction of KIR expression and cytotoxicity assay

NK cells from two donors whose cells did not express the KIR2DL1 gene were incubated in the presence of 25 nM histone deacetylase inhibitor trichostatin A (TSA) or 2 μM of DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5Aza-dC). The cytotoxicity of NK cells before and after induction was determined by a standard europium release assay with a 20:1 (E:T) cell ratio against Cw3-transduced 721.221 cells that lack the ligand for KIR2DL1.

Results

In samples from 73 unrelated individuals, we prospectively evaluated the gene content and expression of the four inhibitory KIRs that had been shown to be important in allogeneic HCT for the treatment of leukemia. Genotyping showed that KIR2DL1 was present in 93% of the individuals, KIR2DL2 in 56%, KIR2DL3 in...
88%, and KIR3DL1 in 92%. Remarkably, in 5 (7%) of the 68 individuals in whom KIR2DL1 gene was present and in 10 (15%) of the 67 in whom KIR3DL1 was present, the corresponding receptor was not found to be expressed by NK cells (1/1000), as determined by flow cytometry analyses (Fig. 1, A and B), with RT-PCR analyses revealing either no band (n = 12) or only a very faint one (n = 3). In contrast, one or both allelic forms of KIR2DL2/KIR2DL3 were expressed by a high proportion of NK cells (15.2–69.3%) in all 73 individuals. However, for those whose genotyping was positive for both KIR2DL2 and KIR2DL3, KIR2DL3 appeared to be preferentially expressed because transcripts of KIR2DL2 were detectable by RT-PCR in only 58% of the individuals, whereas those of KIR2DL3 were found in 100%. In total, results from KIR repertoire assessment by genotyping and those by phenotyping were not in complete agreement in 18 (25%) of the 73 individuals. This disparity was possibly related to allelic polymorphism or epigenetic silencing (25–28).

Because phenotypic polymorphism caused by allelic polymorphism has not been reported for KIR2DL1, we investigated whether DNA methylation or chromatin modification contributed to the lack of gene usage in some donors. We incubated cells from two individuals who did not express KIR2DL1 in hypomethylating agent 5Aza-dC or histone deacetylase inhibitor TSA. Induction of KIR2DL1 expression was seen in both samples after incubation with 5Aza-dC but not after incubation with TSA (Fig. 1, C and D), thus demonstrating that KIR2DL1 can be silenced epigenetically by hypermethylation and that these donors may be misclassified as favorable donors for HLA-C1*0801-negative patients.

Among the samples that tested positive for the expression of a certain KIR gene, the levels of transcripts and surface expression were assessed. Figure 2 shows the heterogeneity in KIR expression in individuals whose NK subsets expressed the KIR gene. A box plot of percentage of NK cells in each donor that were positive for the KIR indicated, as determined by flow cytometry analysis using Abs EB6B, GL183, and DX9. B, Left panel, an RQ-PCR amplification plot. The clusters of curves on the far right are H2O controls. The y-axis indicates the change in fluorescence during the reactions (δ Rn). The cycle threshold (Ct) was defined as the PCR cycle at which the SYBR Green I fluorescence exceeded the threshold for the first time. Thus, the Ct values are inversely proportional to the amount of KIR transcripts present in the sample. Right panel: a zoom-in of the linear phase of three representative samples of which 31.1, 15.7, and 5.7% of the NK cells were positive for KIR3DL1 by flow cytometry analysis (left to right, respectively). The corresponding Ct values for KIR3DL1, as shown from left to right, are 22.5, 23.7, and 24.6. C, Box plot of Ct values as determined by RQ-PCR analysis, A and C. In the box plots, the solid line in the middle of the box represents 50th percentile, the box extends from the 25th to 75th percentile, and the whiskers extend to the upper and lower adjacent values that are <1.5 times the interquartile range. Outside values are not individually plotted.

Figure 3 shows the percentage of samples that by standard RT-PCR analysis expressed the KIR indicated in those who possessed that particular KIR gene. Pairwise genotyping by PCR and phenotyping by RT-PCR analyses were performed on samples from 30 unrelated individuals. Only two KIR members, KIR2DL3 and KIR3DL2, showed 100% agreement, i.e., in all samples in which the KIR gene was present, the corresponding transcripts were also detectable.
the genes (Fig. 3).

...detectable by RT-PCR analyses in the individuals who possessed KIR3DS1 whose NK cell phenotype was compatible with KIR3DL1*004 only 2 of the 12 KIR genes (KIR2DL3).

...tended our investigation to include all 12 KIR gene family members such as KIR2DS2 and KIR2DS3 (7, 31, 32). We then examined the risk of GVHD has been associated with donor-activating role of other KIR family members in HCT. Poor survival or increased risk of relapse was better predicted by an account of the donor KIR repertoire and can, therefore, be alloreactive if the corresponding ligand is absent on the recipient’s cells (12). Indeed, autoreactive KIR2DL2/2DL3+2DL1−3DL1− clones have been found in healthy donors who lack HLA-CAsn80, and NK cells from ~50% of these individuals were cytotoxic against autologous CD34+ cells (33). This corroborated the observation that the risk of relapse in patients with hematologic malignancies, most of which were CD34+, was significantly lower if the donor NK cells expressed an inhibitory KIR (KIR2DL1, KIR2DL2, KIR2DL3, or KIR3DL1) in the absence of its corresponding ligand in the recipient (12). A recent update analysis including additional patients (total n = 51) confirmed that this new missing KIR ligand model is more accurate than the Perugia model (Fig. 4). Together, these results suggest that the “best KIR mismatch” donor can be selected on the basis of the donor’s KIR repertoire and the recipient’s ligand groups (Table I).

In addition to the difference in the definition of KIR mismatch, another level of complexity in studying KIR in HCT involves the use of KIR typing. Many methods are available for assessing the donor KIR repertoire. DNA-based methods allow the analysis of all KIR genes and are logistically attractive, because a single archived (or prospectively collected) DNA sample for HLA typing can also be used for KIR genotyping. However, because of various factors, including allelic polymorphism and epigenetic silencing (25–28, 34, 35), the identification of a certain KIR gene by genotyping does not provide information about the frequency of NK cells expressing that particular KIR. This frequency of KIR expression is crucial because a recent study showed that the size of the subset of NK cells expressing a KIR not engaged by the HLA class I alleles of the patient paralleled the degree of cytotoxicity against the leukemia cells (36). The data provided herein caution the use of genotyping alone for donor KIR assessment because of the lack of gene usage in approximately one-fourth of the individuals for one of the inhibitory KIRs that recognize the three major groups of class I ligands. Even among the samples that tested positive for KIR gene expression, we showed that the levels of transcripts and surface expression varied considerably (Fig. 2). If a certain KIR is not expressed by the donor NK cells before transplantation, then it is not likely to be expressed after transplantation because NK cells typically acquire a donor-specific pattern of KIR expression shortly after CD34+ cell infusion without GVHD prophylaxis (12). Therefore, predicting alloreactivity on the basis of genotyping may not be accurate in these donor-recipient pairs.

### Table I. Suggestion for necessity of donor KIR typing

<table>
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<tr>
<th>Patient HLA Groups</th>
<th>2DL1+</th>
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<td>No</td>
<td>No</td>
</tr>
<tr>
<td>C_{Aas80}/C_{Aas80}/Bw6+</td>
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<td>C_{Aas80}/C_{Asn80}/Bw6+</td>
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<td>C_{Aas80}/C_{Aas80}/Bw6−</td>
<td>No</td>
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<td>Yes</td>
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</table>

* Genotyping alone is inadequate because of low frequency of expression in some donors.
* KIR typing for 2DL2 and 2DL3 is not necessary in general because one or both allelic forms are expressed at high frequency by subsets of NK cells in all donors. No clinical data are available thus far to suggest that the expression of both allelic forms is better than one, or one allelic form is better than the other.
* Patients in this group may benefit from both HLA-B and HLA-C associated KIR mismatch. Prior study demonstrated that NK cell cytotoxicity increased with the number of mismatch pairs (Ref. 12).
* Patients in these groups may benefit from both HLA-B and HLA-C associated KIR mismatch. Prior study demonstrated that NK cell cytotoxicity increased with the number of mismatch pairs (Ref. 12).

* The clinical significance of KIRs other than these four is under investigation.

FIGURE 4.  The risk of relapse was better predicted by an account of missing KIR ligand in the recipient for the donor KIR (receptor-ligand model) than by an account of missing KIR ligand in the recipient for the donor KIR (ligand-ligand model).

...varied considerably (e.g., KIR2DL1 in Fig. 1, A and B). The frequency of KIR2DL1-positive NK cells, as determined by flow cytometry, ranged from 0.4 to 44.7%, that for KIR2DL2/KIR2DL3 from 15.2 to 69.3%, and that for KIR3DL1 from 0.2 to 48.3%. The medians and interquartile ranges are shown by box plots in Fig. 2A. Flow cytometry analysis also showed that the incidence of null phenotype for KIR3DL1 (23%) was more than twice what was expected (i.e., transcripts were easily detectable but surface protein expression was not detected).

To further characterize the heterogeneity of KIR expression by NK cells, we developed a RQ-PCR analysis. The amount of transcripts detectable by RQ-PCR for all four inhibitory KIRs was highly variable among donors (Fig. 2, B and C) and mimicked that seen in flow cytometry analysis (Fig. 2A).

Relative to these four inhibitory KIRs, less is known about the role of other KIR family members in HCT. Poor survival or increased risk of GVHD has been associated with donor-activating KIRs such as KIR2DS2 and KIR2DS3 (7, 31, 32). We then extended our investigation to include all 12 KIR gene family members by performing pairwise genotyping and standard RT-PCR analysis of samples from 30 unrelated individuals. Transcripts of only 2 of the 12 KIR genes (KIR2DL3 and KIR3DL2) were always detectable by RT-PCR analyses in the individuals who possessed the genes (Fig. 3).

**Discussion**

During the period of receptor acquisition after CD34+ cell transplantation, subsets of NK cells may express only one of the inhibitory KIRs in their repertoire and can, therefore, be alloreactive if the corresponding ligand is absent on the recipient’s cells (12). Indeed, autoreactive KIR2DL2/2DL3+2DL1−3DL1− clones have been found in healthy donors who lack HLA-C_{Aas80}, and NK cells from ~50% of these individuals were cytotoxic against autologous CD34+ cells (33). This corroborated the observation that the risk of relapse in patients with hematologic malignancies, most of which were CD34+, was significantly lower if the donor NK cells expressed an inhibitory KIR (KIR2DL1, KIR2DL2, KIR2DL3, or KIR3DL1) in the absence of its corresponding ligand in the recipient (12). A recent update analysis including additional patients (total n = 51) confirmed that this new missing KIR ligand model is more accurate than the Perugia model (Fig. 4). Together, these results suggest that the “best KIR mismatch” donor can be selected on the basis of the donor’s KIR repertoire and the recipient’s ligand groups (Table I).

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KIR expression can be confirmed by using flow cytometry, RNA-based methods, and clonal repertoire assessment. Flow cytometry is relatively easy to perform and allows the estimation of the frequency of single inhibitory KIR-positive cells that are relevant, in terms of alloreactivity toward recipient cells that lack the corresponding ligand (12, 36). Most mAbs recognize both the inhibitory KIR and its activating counterpart, though this may be a less important issue in the future as more specific Abs become available (37). Among the many Abs for the three groups of KIRs that recognize the three major groups of nonubiquitous class I ligands, only DX9 binds to KIR3DL1 but not KIR3DS1 (17, 18) and ECM41 binds to KIR2DL3 but not KIR2DL2/KIR2DS2 (37). Propitiously, the genotype KIR2DL1*2DS1 was found in <3% of the population worldwide and KIR2DL2*2DL3*2DS2 has not been reported (38). Therefore, almost all KIR2DL1-positive samples and all KIR2DL2/2DL3-positive samples by flow cytometry may be assumed to truly express those inhibitory KIRs in subsets of donor NK cells. The interpretation of flow cytometry results can be further complemented as demonstrated herein by RNA-based methods, and clonal repertoire assessment. Flow cytometry is relatively easy to perform and allows the estimation of KIR expression by NK cells among individuals with similar gene content, underscores the important potential of phenotyping in comprehensive KIR evaluation for the selection of stem cell donor or prediction of outcomes outside the transplant setting (39–41).

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Disclosures

The authors have no financial conflict of interest.

References


